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(57) Abstract

The present invention relates to a novel method for the detection of a nucleic acid sequence within a nucleic acid molecule. The method of the invention relies on the combination of nucleic acid protection, ligation of oligonucleotides to the protected nucleic acid molecules and amplification of the ligation products. The detection of the amplified products is advantageously effected by converting the same to the single–stranded form and hybridizing the single–stranded form thereof to an array of single–stranded nucleic acid molecules of at least partially predetermined sequence fixed to a solid support. The solid support is preferably a chip. Detection of hybridized molecules can be effected according to conventional methods. The present invention additionally relates to a kit for carrying out the method of the invention.

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Method for the Detection of Nucleic Acid Sequences

The present invention relates to a novel method for the detection of a nucleic acid sequence within a nucleic acid molecule. The method of the invention relies on the combination of nucleic acid protection, ligation of oligonucleotides to the protected nucleic acid molecules and amplification of the ligation products. The detection of the amplified products is advantageously effected by converting the same to the single-stranded form and hybridizing the single-stranded form thereof to an array of single-stranded nucleic acid molecules of at least partially predetermined sequence fixed to a solid support. The solid support is preferably a chip. Detection of hybridized molecules can be effected according to conventional methods. The present invention additionally relates to a kit for carrying out the method of the invention.

The detection of nucleic acid sequences within biological samples becomes increasingly important. With the advent of the polymerase chain technology (PCR) (see, for example, Saiki et al, Science 239 (1988) 487-491), a significant advance in the role of molecular biology in diagnosis was achieved. In addition, a growing number of genes has become available, mutations in which are related to human diseases. For example, Scherzinger et al, Cell 90 (1987), 549-558, have demonstrated a correlation between the number of glutamine repeats in the huntingtin gene and a phenotype correlated with Huntington's Disease. It is expected that further human diseases will be directly linked to genetic disorders in the future. A number of methods are available that allow the detection of genetic disorders such as point mutations, deletions or duplications. Such methods include PCR, RFLP analysis or Southern blotting in combination with nucleic acid hybridization. Often, these methods are, however, still rather laborious or allow a detection of only one nucleic acid sequence at a time. For

example, the classic PCR method, as a rule, requires specific primers for each DNA sequence that is to be analyzed. An improvement of the classic PCR is represented by the so-called multiplex PCR which has, however, turned out not to be a very robust technique. Thus, oligonucleotide primer pairs as used for PCR show different efficiencies for priming and amplifying a DNA sequence. If several primer pairs with different priming features are used in the same reaction, the rate of misprimings is increased and the amount of specific PCR products is decreased. When using "strong" (i.e. very efficient) primers together with weak (i.e. not very efficient) primers only the "strong" primers may yield a detectable product. Furthermore do different assay conditions (Mg^{2+} concentration, template concentration, primer concentration, annealing temperature, even different batches of DNA-polymerase) show a great influence on the amount of different PCR products.

Accordingly, the technical problem underlying the present invention was to develop a method that allows for the easy detection of specific nucleic acid sequences within the sample and which further can be carried out at a rather low cost. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Thus, the present invention relates to a method for the detection of a nucleic acid sequence within a nucleic acid molecule comprising the steps of

(a) hybridizing single-stranded nucleic acid to one or more single-stranded nucleic acid probes;

(b) removing non-hybridized nucleic acid from the product of step (a);

(c) converting the hybrid obtained in step (b) into single-stranded form;

(d) ligating oligonucleotides to the single-stranded nucleic acid obtained in step (c); or

(c') ligating oligonucleotides to the hybrid obtained in step (b);

(d') converting the ligation product of step (c') into single-stranded form;

(e) carrying out an amplification reaction with the product of step (d) or (d') using primers that hybridize to said oligonucleotides or transcribing the product of step (d) or (c); and

(f) detecting the product of step (e).

The method of the invention allows, by using only one pair of e.g. complementary oligonucleotides and two primers (subsequently also termed "universal primers") hybridizing thereto, the amplification and subsequent detection of virtually any nucleic acid sequence that forms a double-stranded nucleic acid hybrid with a chosen probe. It is particularly preferred that one and the same oligonucleotide is used for ligation to the 5'-end as well as the 3'-end and also as the primer. Examples of such oligonucleotides are oligonucleotides comprising palindromic sequences. Thus, optionally, one oligonucleotide is sufficient for all steps to be carried out in the method of the invention.

In accordance with the present invention, the term "nucleic acid molecule" comprises also any feasible derivative of a nucleic acid to which a nucleic acid probe may hybridize. In addition, the nucleic acid probe may be any derivative of a nucleic acid capable of hybridizing to said nucleic acid molecule.

In other embodiments, the primers employed in the method of the invention may not be identical with the oligonucleotides used for ligation. Nevertheless, advantageously the same primer may be used for hybridization to the 3'-end and the 5'-end of the target oligonucleotide, depending on the sequence of said target.

Consequently, the need for preparing a larger number of different primers or primer pairs for the analysis of biological samples falls away. If desired, oligonucleotides or pairs of oligonucleotides with different nucleic acid sequences may be employed and manipulated according to conventional methods for ligation either to the 5'- or 3'- ends of the amplified nucleic acid molecules (see, for example, Sambrook et al, "Molecular Cloning, A Laboratorial Manual" CSH Press, Cold Spring Harbor, (1989)). Naturally, more than one single-stranded nucleic

acid probe can be hybridized to said nucleic acid. Also, the sample may comprise more than one different nucleic acid molecule, such as a pool of different nucleic acid molecules.

The sample may be obtained from any biological or synthetic source. Preferably, the sample is taken from a natural source such as blood, serum, stool, sputum, tissue etc. Applied to samples from natural sources, the method of the invention may be used for the detection of genetic disorders indicative of human or animal diseases.

In its broadest aspect, the method of the invention envisages, with respect to the timing of the ligation of the oligonucleotides, two alternatives. Either, the oligonucleotides are ligated to single-stranded protected nucleic acid molecules after removal of the protecting strand or they are, preferably in double stranded form, ligated to the double-stranded hybridization product. In both cases, non-hybridized single-stranded nucleic acid is removed prior to the ligation. Further, either single-stranded or double-stranded oligonucleotides may be ligated to the product according to the first and to the second alternative. As regards the first alternative, it is to be noted that the protecting strands to be removed may be either the strand that was originally present in the sample or the single-stranded probe.

As regards the nature and nucleotide sequence of the oligonucleotides the following should be noted: if said oligonucleotides are ligated as double-stranded complementary nucleic acid molecules, it is advantageous to ligate them first to the 5'-end and subsequently to the 3'-end of the protected nucleic acid molecules, or vice versa. Advantageously and preferably, the double-stranded oligonucleotides comprise a recognition and cleavage site for a restriction endonuclease.

As regards the embodiment comprising the transcription of the product in step (e), the following should be noted: in this embodiment, at least one of the oligonucleotides to be ligated to the protected or protecting nucleotide sequence comprises a promoter for a polymerase, preferably a RNA polymerase. Upon ligation, the corresponding transcript can be produced and visualized according to standard protocols.

By setting the conditions for hybridization, the person skilled in the art can determine if strictly complementary sequences or sequences with a higher or lower degree of homology are to be detected. The setting of conditions is well within the skill of the artisan and to be determined according to protocols described, for example, in Sambrook, loc. cit. or Hames and Higgins, "Nucleic acid hybridization, a practical approach", IRL Press, Oxford (1985). Thus, the detection of only specifically hybridizing sequences will usually require stringent hybridization and washing conditions such as 0.1xSSC, 0.1% SDS at 65° C. Non-stringent hybridization conditions for the detection of homologous and not exactly complementary sequences may be set at 6xSSC, 1% SDS at 65°C. As is well known, the length of the probe and the composition of the nucleic acid to be determined constitute further parameters of the hybridization conditions.

For the other steps required in the method of the invention, the person skilled in the art is in the position to practice them by reverting, for example, to conventional protocols. Thus, the removal of single-stranded nucleic acid that did not form a hybridization product with the oligo- or polynucleotide used as a probe as well as of probes that did not hybridize to any target nucleic acid sequence can be effected according to protocols described, for example, in Sambrook et al, loc. cit. For example, single-stranded nucleic acid such as mRNA may be removed by S1 nuclease or mung bean nuclease digestion. Double-stranded nucleic acid such as DNA may be removed by employing protocols using λ-exonuclease. Conventional protocols may also be employed for the ligation step, the conversion of double-stranded nucleic acid into single-stranded nucleic acid or for the amplification

which may conveniently be carried out by using PCR technology, strand displacement amplification, linker ligation combined with *in vitro* transcription or techniques comprising rolling circle amplification if the protector or the protected molecule is a circular molecule. Thus, in a template dependent ligation, T4-DNA ligase may advantageously be used. Template independent ligation would require a different enzymatic activity such as conferred by T4-RNA ligase. Further, for example, the conversion of double-stranded into single-stranded nucleic acid may be done by base or heat denaturation. The person skilled in the art is further capable of converting the complete double-stranded nucleic acid within a sample into a single-stranded form, if this is desired. Suitable protocols include, alone or in combination, strand displacement reactions by polymerase, chemical or enzymatic degradation of either strand, affinity chromatography if the introduced primer is labeled and chemical denaturation of (hetero-)duplex nucleic acid molecules. This measure will also prevent the formation of double-stranded hybrids within the nucleic acid to be analyzed to which later oligonucleotides would be ligated, thus giving rise to false positive results. Double-stranded template dependent ligation of 5'-primer oligonucleotides may also be performed in a cyclic ligation reaction using thermostable ligases (e.g. Taq Ligase). For this purpose, different types of single-stranded adaptor molecules may be used which are complementary to the amplification primer oligonucleotides and terminally complementary to the protected oligonucleotides. The 5'- and 3'-ends of the adaptor and the first primer oligonucleotide may in this embodiment be connected forming a hairpin loop.

Whereas it is possible to change reaction vials after each step, it is preferred to carry out at least steps (b) to (e) in one reaction vessel, preferably in an Eppendorf tube.

It is preferred that the nucleic acid to be detected and/or the nucleic acid probe is comprised of RNA, DNA or PNA. Examples of RNA are rRNA and mRNA, of

DNA, cDNA and genomic DNA. Any of the above preferred embodiments may also be (semi)synthetically produced.

Whereas a number of options are available to the person skilled in the art for removal of the non-hybridized nucleic acid in step (b), it is preferred that said removal is effected by exonuclease activity, such as λ-exonuclease activity optionally in combination with (restriction) endonuclease activity, by affinity chromatography using, for example, antibodies specific for double-stranded nucleic acid coupled to a conventional matrix or by gel-electrophoresis or HPLC. It is also envisaged in accordance with the present invention that any one or the combination of the above mentioned nuclease activities are combined with affinity chromatography, gel-electrophoresis and/or HPLC in order to remove non-hybridized nucleic acid in step (b). As used in accordance with the present invention, the phrase "removing non-hybridized nucleic acid" evidently also comprises the removal of single-stranded regions of said nucleic acid that have not formed a nucleic acid hybrid with said one or more nucleic acid probes.

In an additional preferred embodiment of the method of the invention, the oligonucleotides used for ligation are masked at their 3'-ends. Masking the 3'- or 5'-ends of said oligonucleotides in this embodiment of the invention is required if template independent ligation method as with T4-RNA-ligase is used. For example, one would use one primer which has no 5'-phosphate and a normal 3'-hydroxy group which is ligated to the 5'-phosphate group of the nucleic acid to be analyzed thereby forming a phosphodiesterbond. For the primer ligation to the 3'-hydroxy group of said analyte one might use a 3'-blocked oligonucleotide (e.g. an oligonucleotide carrying an amino block) with an intact 5'-phosphate group.
3'- as well as 5'- primer oligonucleotides are able to form a dimer. The dimers have a 5'-hydroxy group and a 3'-blocked end unable to form polymers of higher order. The primer ligation steps to the 3'-end and to the 5'-end have, in these embodiments, therefore to be carried out sequentially in separate reactions to avoid primer-dimer formations. Alternatively, the oligonucleotides used for ligation

are designed to form, upon dimer formation, a restriction site for an endonuclease which can subsequently be cleaved.

Thermostable restriction enzymes can be used in a PCR amplification reaction for cleaving the primer dimers. The following is an example of this embodiment. The 5'-oligomer to be ligated to the 5'-end of the protected nucleic acid molecule has the sequence

5'-OH-GCACCGCGGAATTCTCGAGGACAA-OH-3'

whereas the 3'-oligomer has the following sequence:

5'-P-AGTCCGTGGCGCCTTAAGAGCTC-3'-X

X denotes the amino block. Upon dimer formation, the following sequence is established

5'-OH-GCACCGCGGAATTCTCGAG**GACAAAGTC**CGTGGCGCCTTAAGAGCTC -3'-OH

wherein a TthI restriction site is emphasized by bold letters. Final amplification may be effected using single-primer oligonucleotides of the following sequence:

5'-OH-GCACCGCGGAATTCTCGAG-3'-OH

Cleavage with TthI should be effected early, i.e. once the first double-strands have been formed.

It is further preferred that, in the amplification step (e), labeled nucleotides are incorporated into the amplification product. This embodiment of the method of the invention has the advantage that the amplification product is directly visible in

detection step (f) without the need for an additional detectable means. Conveniently, the label is a radioactive label, a fluorochrome, a bioluminescent label, a chemiluminescent label, a hapten, an enzyme such as horseradish peroxidase or a chelator for the detection of bound metals.

As has been mentioned herein above, the detection step (f) can be carried out by a variety of conventional protocols. Such methods include filter hybridization, PCR-ELISA, mass spectroscopy and dot blot assays. For example, if the protecting nucleic acid molecules are designed to have a distinguishable length, the protected nucleic acid sequences/molecules have a distinct length and mass. Accordingly, such molecules, in a wide range of masses, will be appropriate for analysis by mass spectroscopy. In addition, once the nucleic acid sequence in the sample has been determined, it can be verified by nucleic acid sequencing if an exactly complementary nucleic acid sequence was searched for or it can be determined if a homologous sequence was to be identified.

For detecting the amplification product in step (f), the following detailed protocol is particularly preferred:

(f') converting the amplified product of step (e) into single-stranded form;

(f") contacting the single-stranded nucleic acid molecules obtained in step (f') with an array of single-stranded nucleic acid molecules with at least partially predetermined sequences attached to a solid support under conditions that allow the formation of hybrids between said single-stranded nucleic acid molecules and nucleic acid molecules with said at least partially predetermined sequence to occur; and

(f"') detecting the formation of hybrids formed in step (f").

Again, the conversion of the amplified product into single-stranded form can be effected by conventional protocols that have been outlined above.

Also, the hybridization conditions in step (f") will be determined along the various schemes delineated herein above. The single-stranded nucleic acid molecules

arranged in array form on a solid support may have a fully or partially predetermined sequence. They may be of natural, synthetic or semi-synthetic origin. Attachment to the solid support may be effected according to conventional protocols, for example, via a biotin-avidin bridge.

The detection of hybrids in step (f''') can be carried out by a variety of means, usually depending on the labeling that is employed. For example, if the amplification in step (e) is effected by using fluorochrome-labeled oligonucleotides, said detection may be by visual means, for example with a correspondingly equipped microscope. Other options include capillary and gel electrophoresis, HPLC, mass spectroscopy, nucleic acid hybridization and nucleic acid sequencing. One preferred embodiment also applicable to the other embodiments of the method referred to above, provides for the detection by using an anti-double-stranded DNA antibody. Said antibody may either be detectably labeled or it may be detected using, for example, a tertiary antibody that is detectably labeled. Another preferred embodiment employs for detection is hybridization with a detectably labelled probe. Any detection method discussed above may be assisted by computer technology.

The array referred to above may be any two- or three dimensional arrangement of molecules.

It is particularly preferred that the array is in grid form.

It is further particularly preferred that said solid support is a chip. In one embodiment, this chip consists of inorganic substances (glass, silica and others) or of organic substances (for example polymers) or is a hybrid consisting of different substances or layers of different substances (plastic coated glass, for example, silane coated silica). The oligonucleotide bound to this chip could be directly bound by covalent binding or indirectly via hapten interactions (e.g. a biotin-avidin bridge).

In a further preferred embodiment of the method of the invention, after step (b) and prior to step (e), the following step is carried out

(b') cleaving a mismatch contained in the hybrid obtained in step (b). The step characterizing this embodiment may be carried out prior to or after any of steps (c), (c'), (d), and (d'). Cleaving of this matches is conveniently done by the employment of appropriate enzymes such as Cleavase™ (Boehringer Mannheim). Upon cleavage of one or optionally both of the hybridized strands, an amplification product will not form any longer. This specific embodiment of the invention will find wide range of applications in the demonstration of the presence of specific mRNA sequences or of mutations in a genomic sequences related, for example, to cancer. Thus, it is known Ras sequences contributing to the formation of tumorous cells carry one specific mutation in their coding sequence. Preparing a protecting oligonucleotide that is exactly complementary to the mutated sequence will result in the amplification of that mutated sequence. Accordingly, cancerous cells can be identified in the sample by applying the method of the invention. If the protecting oligomer is hybridized only to wildtype sequences, a mismatch will be formed and, upon cleavage, no amplification product will be produced. Accordingly, the lack of appearance of an amplification product is indicative of the absence of turnorous cells. The basic teaching underlying this embodiment can, of course, be applied to the person skilled in the art to a wide variety of purposes.

Further, the present invention relates to a kit comprising

(a) matrix bound protecting nucleic acid molecules; and

(b) universal primer oligonucleotides.

The matrix may be any conventional matrix such as polystyrol or magnetic beads or a chip. The person skilled in the art is able to design the protecting nucleic acid

molecules according to the specific target molecules that are to be analyzed. Advantageously, the matrix comprises a mixture of different nucleic acid molecules which may be of entirely different origin. Advantageously, the kit of the invention further comprises, besides the universal primer oligonucleotide to be used for optionally both backward and forward priming as well as optionally for ligation to the protected target nucleic acid molecule, hybridization buffer(s), nuclease(s) such as nuclease S1 or mung bean nuclease, nuclease buffers, ligase(s) and ligation buffer(s), DNA polymerase(s) and polymerase buffer(s) suitable for the amplification of the ligation products and/or a control nucleic acid the antisense sequence of which is also bound to the above referenced matrix. The components of the kit of the invention may be packaged in containers such as vials, optionally in buffers and/or solutions. If appropriate, one or more of said components may be packaged in one and the same container.

The kit may be advantageously used for carrying out the method of the invention.

The references cited in the specification are herewith incorporated by reference.

The figures show:

Figure 1: Schematic overview of one preferred embodiment of the method of the invention. Nucleic acids are protected by a protector molecule from degradation with nucleases. After removal of degradation products primers are ligated to each end of the protected nucleic acids and the protected nucleic acids are amplified by a universal PCR (one primer pair) for all protected nucleic acid fragments. Subsequently, the amplification products are visualized by various methods.

Figure 2: One further embodiment of the invention drawn schematically and comprising the amplification of nucleic acid fragments after the protection reaction using matrix bound protector molecules.

(A) nucleic acids capture

(B) degradation of non-protected nucleic acids

(C) ligation of primers

(D) universal PCR.

Figure 3: Universal PCR for the amplification of nucleic acid fragments after the protection reaction.

P1 = primer 1; cP1 = complementary to primer P1; P2 = primer 2; cP2 = complementary to primer P2

In the first ligation step a primer oligonucleotide is ligated to the 5'-end of the protected molecule. In the second ligation step an other oligonucleotide is ligated to the 3'-end of the protected DNA-molecule.

(A) Synthesis of the one strand with one primer.

(B) Synthesis of the one strand with the other primer. The possibility of primer dimers having their origin in the ligation reactions if there are contaminating DNA molecules of one species ligated to the other one can be circumvented if

(C) 1. there are no contaminating molecules to be ligated together; or if

2. the sequences of the ligated primer molecules form a recognition site for a restriction enzyme (indicated in thin bars) and can be cleaved in a subsequent reaction D (e.g. in the amplification reaction if a thermostable restriction endonuclease like TthI is present in the buffer).

Figure 4: Single primer PCR for "universal PCR" amplification. (P1.1 = single primer 1; cP1.1 = complementary to single primer P1.1; P1.2 = single primer 2; cP1.2 = complementary to single primer P1.2)

In the first ligation step a primer oligonucleotide is ligated to the 5'-end of the protected molecule. In the second ligation step a oligonucleotide complementary to the 5'-oligonucleotide is ligated to the protected DNA-molecule. With an amplification primer complementary to one of the ligated terminal oligonucleotides it is possible to amplify the protected DNA fragments.

(A) Synthesis of the one strand with the same primer as the other strand, since the complementary sequence is produced in each synthesis cycle.

(B) The possibility of primer dimers having their origin in the ligation reactions if there are contaminating DNA molecules of one species ligated to the other one can be circumvented if

(C) 1. there are no contaminating molecules to be ligated together; or if

2. the sequences of the ligated primer molecules are designed to form a recognition site for a restriction enzyme (indicated in double bars) and therefore can be cleaved in a subsequent reaction D (e.g. in the amplification reaction if a thermostable restriction endonucleases like TthI is present in the buffer).

The Examples illustrate the invention.

Example 1

Analysis of mRNA for the presence of specific target sequences.

Insulin specific mRNA was prepared from freshly obtained pancreatic tissue according to standard procedures. The mRNA was dissolved in hybridization buffer and insulin specific oligonucleotides of the following sequence:

5'TGGGGCTGCTCTCTCCAAGGTAGGAAGGGGACACCCTGGCCGGTCAAGC
CTGGAGGGTGTTGGGTGCTCTCTCTGGAGGGCAATGTCTAGGCCCCTCGAG
-3'

to be used as protectors which were bound to matrix beeds were added. The hybridization was carried out under constant agitation of the hybridization mixture for 16 hours at 65°C. Subsequently, the hybridization solution was centrifuged and unhybridized RNA was removed by washing the pellet and then repeating the centrifugation and washing steps three times. Single-stranded nucleic acid was subsequently removed by using either S1 nuclease or mung bean nuclease and incubating the mixture at 37°C for an hour. Afterwards, the nucleases were removed by alternative washing steps with conventional washing buffers and centrifugation. The remaining hybrids were denatured by treatment either under heating conditions or with low salt buffer. The protecting nucleic acid was removed by centrifugation. The supernatant was transferred to a new reaction vessel. To the supernatant conventional ligation buffer was added. The first oligonucleotide was added and ligated for one hour with T4-RNA ligase. Not ligated oligonucleotide was removed by conventional washing steps. Subsequently, the second primer was ligated using the same ligation conditions as above. Again, the not ligated oligonucleotide was removed by conventional washing steps. The ligated nucleic acid was precipitated by adding ethanol and salt using conventional conditions. To the precipitated nucleic acid a conventional RT-PCR buffer mix was added. The nucleic acid was amplified employing 30 PCR cycles in the presence of fluorescence labeled primer oligonucleotides complementary to the oligonucleotides ligated to the target sequence. The amplified product was analyzed with a β-Imager (Molecular Dynamics).

Example 2

Detection of changes in gene-expression in amplified regions of chromosome 3 in tumor cells.

Tumor cell lines containing an amplification in chromosome 3 (3q27-3q28) were analyzed for the overexpression of the following candidate oncogenes: BCL6, DLGH, DVL3, EIF4G, HSERM, HSFGF12, HSLPP, HUMP, HUMSOMI, RFC4. 5' phosphorylated single-stranded DNA protectors complementary to the mRNA transcribed from said oncogenes of different distinguishable length in polyacrylamide gels were constructed using Genbank data and conventional oligonucleotide synthesis techniques. RNA from the tumor cell lines was prepared by acidic guanidinethiocyanate/phenol/chloroform extraction. S1 protection was carried out by using an aliquot of the designed oligonucleotides and 20 µg of total RNA. Hybridization was performed overnight in PIPES buffer. After hybridization the non-hybridized RNA and DNA was removed by nuclease S1 treatment and subsequently the DNA protectors were removed by DNA specific nuclease treatment (RNAse free DNAse I). After precipitation the RNA was dissolved in ligation buffer and the primer sequences were subsequently added by T4-RNA ligase in two distinct 5' and 3' ligation steps. Hence all the protected RNA sequences contain the same flanking regions which could be used for amplification by Tth polymerase in a one tube RT-PCR reaction. Because of identical primers of all the sequences, the PCR reaction amplified the RNA in the same ratio as it was present in the cells.

Hence all the problems of multiplex PCR could be avoided. Detection of the amplification products was done by agarose gel electrophoresis and ethidium bromide staining.

Claims

1. A method for the detection of a nucleic acid sequence within a nucleic acid molecule comprising the steps of
   (a) hybridizing single-stranded nucleic acid to one or more single-stranded nucleic acid probes;
   (b) removing non-hybridized nucleic acid from the product of step (a);
   (c) converting the hybrid obtained in step (b) into single-stranded from;
   (d) ligating oligonucleotides to the single-stranded nucleic acid obtained in step (c); or
   (c') ligating oligonucleotides to the hybrid obtained in step (b);
   (d') converting the ligation product of step (c') into single-stranded form;
   (e) carrying out an amplification reaction with the product of step (d) or (d') using primers that hybridize to said oligonucleotides or transcribing the product of step (d) or (c); and
   (f) detecting the product of step (e).

2. The method of claim 1 wherein said nucleic acid is RNA.

3. The method of claim 1 or 2 wherein said nucleic acid is DNA.

4. The method of any one of claims 1 to 3 wherein said nucleic acid is PNA.

5. The method of any one of claims 1 to 4 wherein the removal of non-hybridized nucleic acid in step (b) is effected by nucleases activity, affinity chromatography or gel electrophoresis.

6. The method of anyone of claims 1 to 5 wherein said oligonucleotides are masked at their 3' ends.

7. The method of any one of claims 1 to 6 wherein in the amplification step (e) labeled nucleotides are incorporated into the amplification product.

8. The method of any one of claims 1 to 7 wherein the detection of step (f) comprises

(f') converting the amplified product of step (e) into single-stranded form;

(f") contacting the single-stranded nucleic acid molecules obtained in step (f') with an array of single-stranded nucleic acid molecules with at least partially predetermined sequences attached to a solid support under conditions that allow the formation of hybrids between said single-stranded nucleic acid molecules and nucleic acid molecules with said at least partially predetermined sequence to occur; and

(f'") detecting the formation of hybrids formed in step (f").

9. The method of claim 8 wherein said array is in grid form.

10. The method of claims 8 or 9 wherein said solid support is a chip.

11. The method of any one of claims 1 to 8 further comprising, after step (b) and prior to step (e) the following step:

(b') cleaving a mismatch contained in the hybrid obtained in step (b).

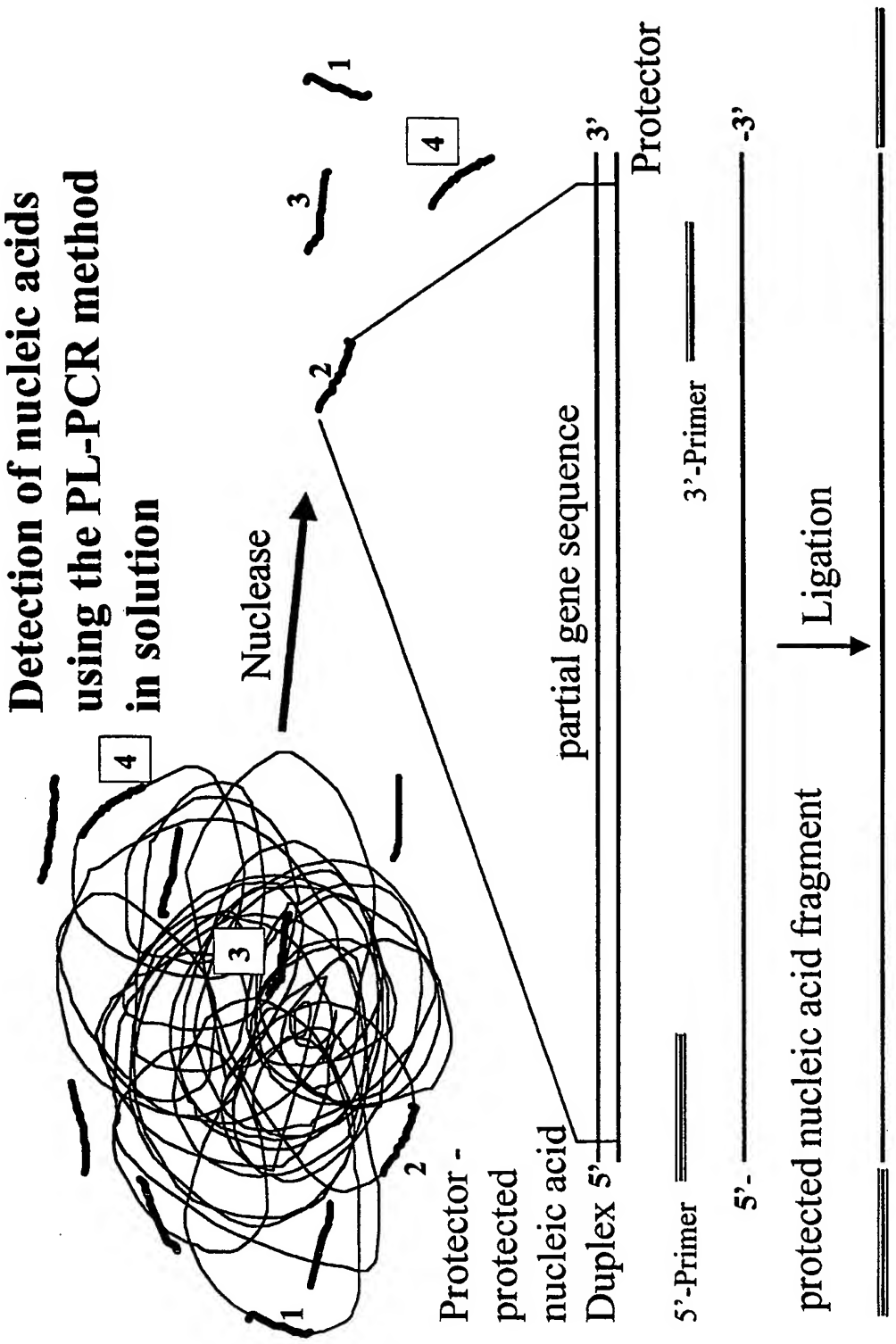
12. The method of any one of claims 1 to 11 wherein said detecting in step (f) is effected by using an anti-double-stranded-DNA-antibody or by hybridisation using a detectably labelled probe.

13. The method of claim 12 wherein said antibody is detectably labeled.

14. Kit comprising

(a) matrix-bound protecting nucleic acid molecules; and

(b) universal primer oligonucleotides.


Detection of nucleic acids
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Nuclease
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protected
nucleic acid
Duplex 5'
partial gene sequence
3'
Protector
5'-Primer
3'-Primer
5'-
-3'
protected nucleic acid fragment
Ligation
1
2
3
4

Fig. 1

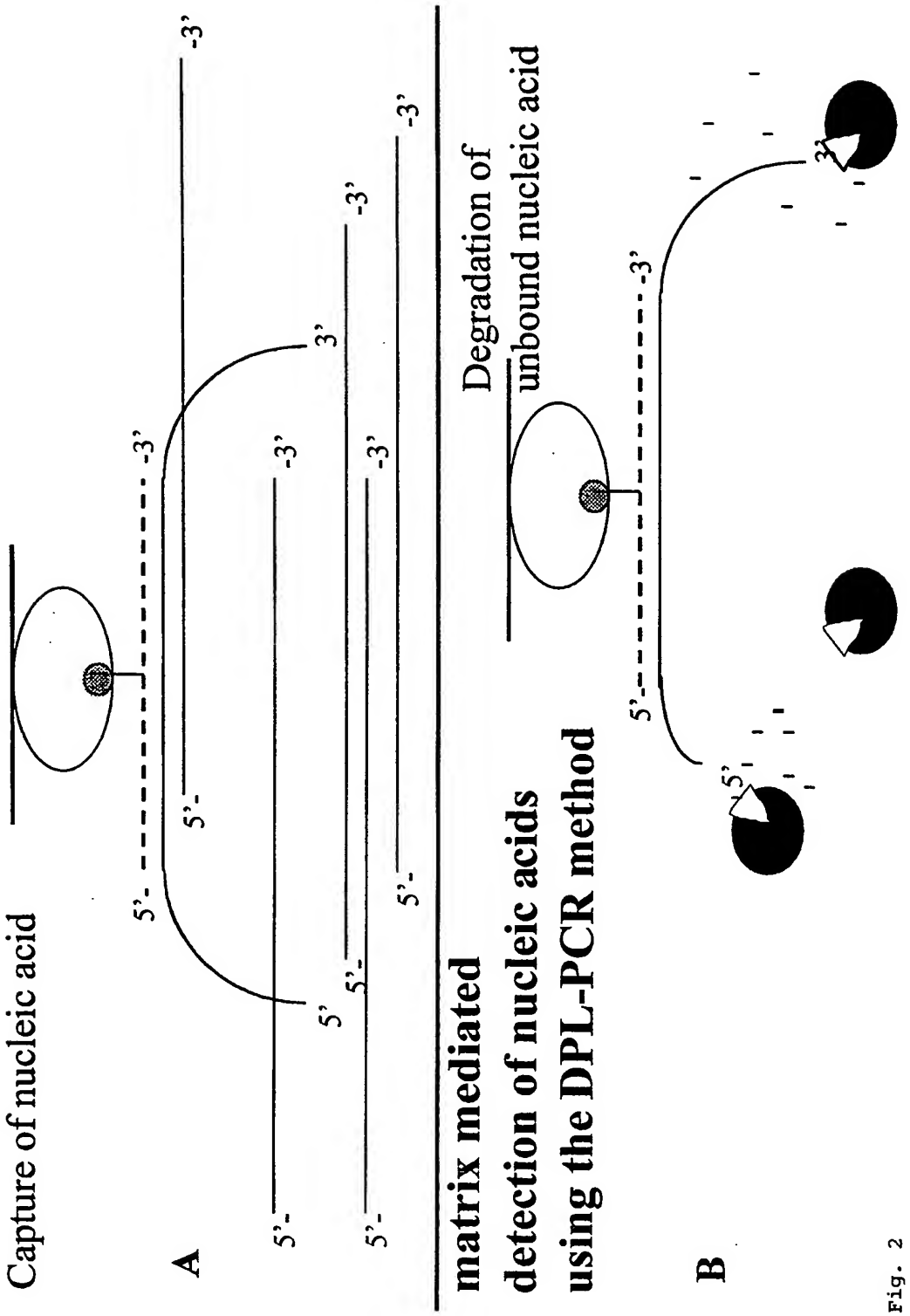
Capture of nucleic acid
A
matrix mediated
detection of nucleic acids
using the DPL-PCR method
Degradation of
unbound nucleic acid
B
5'-
-3'
5'
3'

Fig. 2

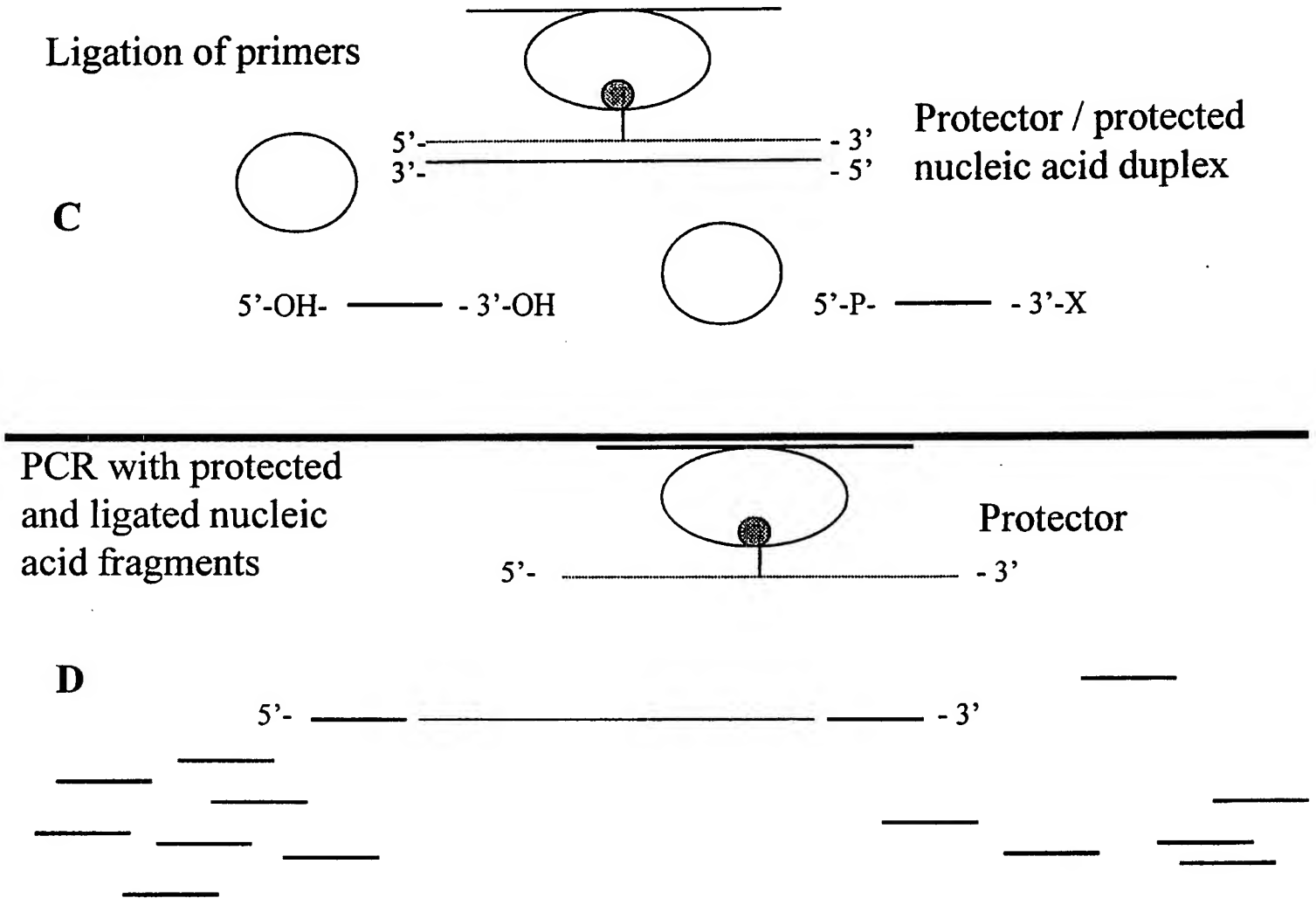
Ligation of primers
5'-
- 3'
3'-
- 5'
Protector / protected nucleic acid duplex
C
5'-OH-
- 3'-OH
5'-P-
- 3'-X
PCR with protected and ligated nucleic acid fragments
5'-
- 3'
Protector
D
5'-
- 3'

Fig. 2 cont.

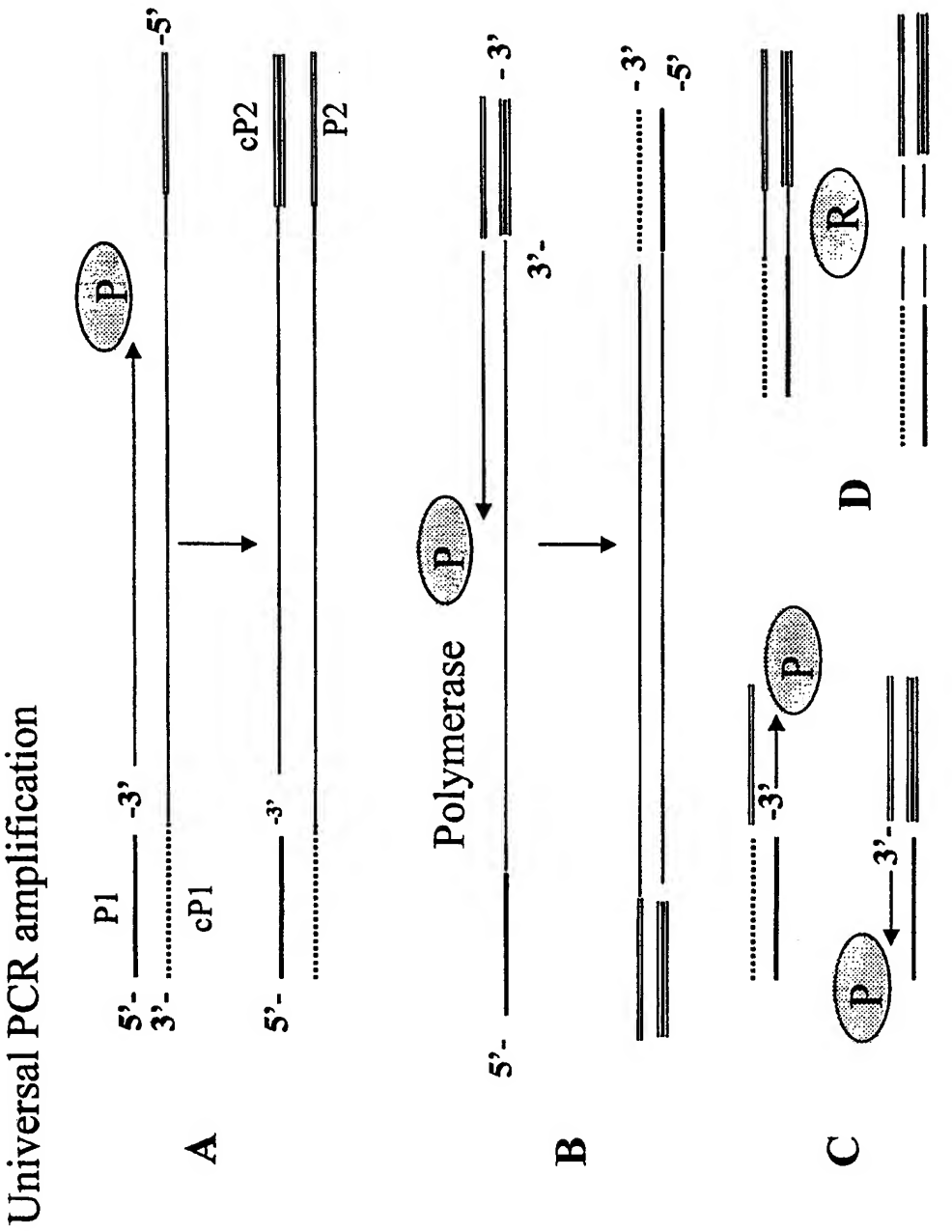

Fig. 3

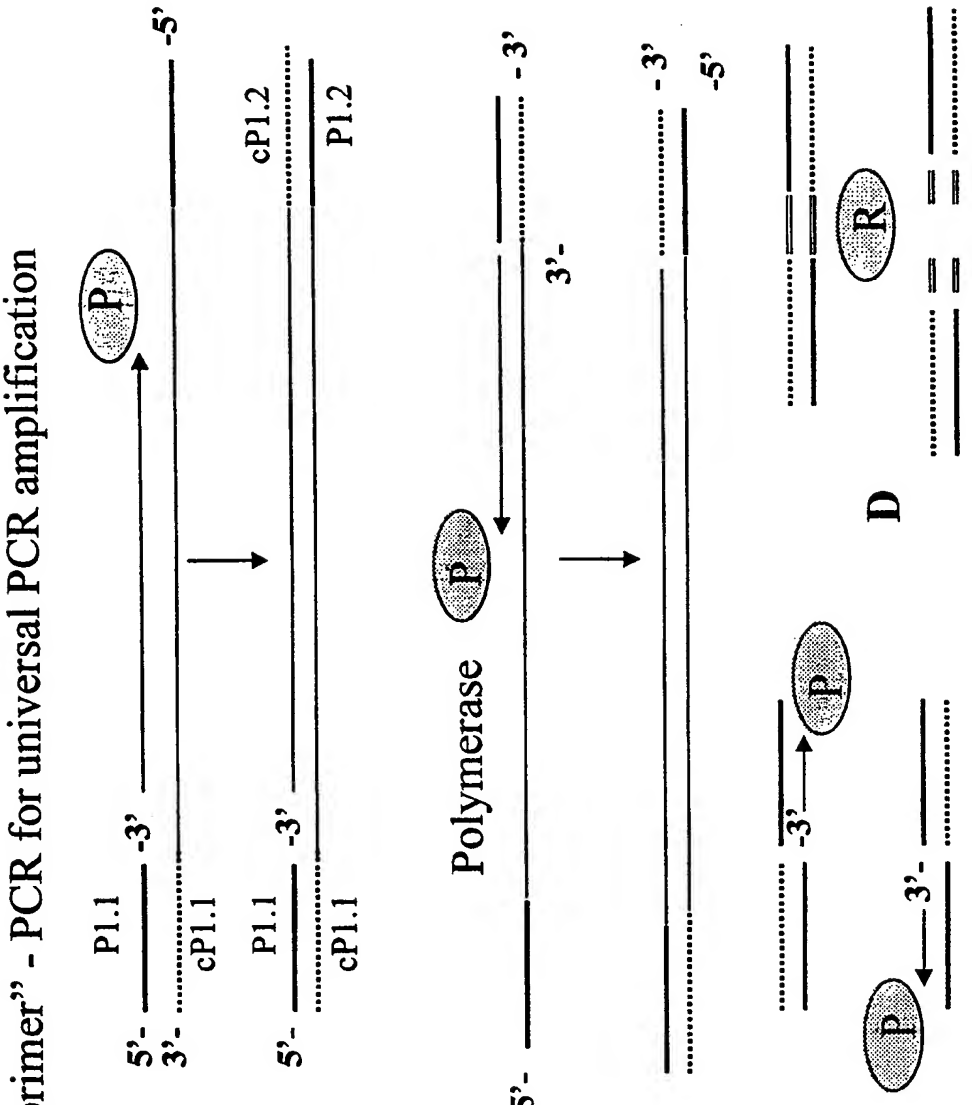
"single primer" - PCR for universal PCR amplification
P1.1
5'-
3'-
-3'
-5'
cP1.1
A
cP1.2
P1.2
Polymerase
P
3'-
-3'
B
C
D
R

Fig. 4

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(57) Abstract

The present invention relates to a novel method for the detection of a nucleic acid sequence within a nucleic acid molecule. The method of the invention relies on the combination of nucleic acid protection, ligation of oligonucleotides to the protected nucleic acid molecules and amplification of the ligation products. The detection of the amplified products is advantageously effected by converting the same to the single–stranded form and hybridizing the single–stranded form thereof to an array of single–stranded nucleic acid molecules of at least partially predetermined sequence fixed to a solid support. The solid support is preferably a chip. Detection of hybridized molecules can be effected according to conventional methods. The present invention additionally relates to a kit for carrying out the method of the invention.

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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30 August 1999	07/09/1999

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